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Expression of CDK7, Cyclin H, and MAT1 Is Elevated in Breast Cancer and Is Prognostic in Estrogen Receptor–Positive Breast Cancer

Hetal Patel[1], **Rezvan Abduljabbar**[2], **Chun-Fui Lai**[1], **Manikandan Periyasamy**[1], **Alison Harrod**[1], **Carolina Gemma**[1], **Jennifer H. Steel**[1], **Naina Patel**[1], **Claudia Busonero**[1], **Dena Jerjees**[2], **Judit Remenyi**[3], **Sally Smith**[4], **Jennifer J. Gomm**[4], **Luca Magnani**[1], **Balázs Győrffy**[5,6], **Louise J. Jones**[4], **Frances Fuller-Pace**[3], **Sami Shousha**[7], **Laki Buluwela**[1], **Emad A. Rakha**[2], **Ian O. Ellis**[2], **R. Charles Coombes**[1], and **Simak Ali**[1]

[1]Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, London, United Kingdom

[2]Department of Cancer and Stem Cells, School of Medicine, University of Nottingham, Nottingham, United Kingdom

[3]Division of Cancer Research, University of Dundee, Ninewells Hospital and Medical School, Dundee, United Kingdom

[4]Centre for Tumour Biology, Barts Cancer Institute, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London, United Kingdom

[5]MTA TTK Lenduület Cancer Bio-marker Research Group, Budapest, Hungary

[6]2nd Department of Pediatrics, Semmelweis University, Budapest, Hungary

[7]Department of Histopathology, Charing Cross Hospital, Imperial College London, United Kingdom

Corresponding Author: Simak Ali, Imperial College London, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, United Kingdom. Phone: 02075942811; simak.ali@imperial.ac.uk.

Disclosure of Potential Conflicts of Interest

S. Ali and R.C. Coombes are listed as co-inventors on a patent on a CDK7 inhibitor that is owned by Imperial College London. S. Ali is a consultant for Carrick Therapeutics plc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L. Buluwela, E.A. Rakha, R.C. Coombes, S. Ali

Development of methodology: H. Patel, R. Abduljabbar, C.-F. Lai, M. Periyasamy, S. Shousha, L. Buluwela, S. Ali

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Patel, R. Abduljabbar, A. Harrod, J.H. Steel, S. Smith, J.J. Gomm, L.J. Jones, F. Fuller-Pace, S. Shousha, E.A. Rakha, I.O. Ellis, R.C. Coombes, S. Ali

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Patel, R. Abduljabbar, C. Gemma, D. Jerjees, L. Magnani, B. Győrffy, L. Buluwela, E.A. Rakha, I.O. Ellis, S. Ali

Writing, review, and/or revision of the manuscript: H. Patel, R. Abduljabbar, L. Magnani, B. Győrffy, F. Fuller-Pace, L. Buluwela, I.O. Ellis, R.C. Coombes, S. Ali

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Patel, R. Abduljabbar, N. Patel, D. Jerjees, E.A. Rakha, S. Ali

Study supervision: H. Patel, R.C. Coombes, S. Ali

Other (helped other authors performing experiments): C. Busonero

Other (prepared and provided the patient tumor RNAs for analysis): J. Remenyi

Other (provided patient tumor RNAs and reviewed article): F. Fuller-Pace

Abstract

Purpose—CDK-activating kinase (CAK) is required for the regulation of the cell cycle and is a trimeric complex consisting of cyclin-dependent kinase 7 (CDK7), Cyclin H, and the accessory protein, MAT1. CDK7 also plays a critical role in regulating transcription, primarily by phosphorylating RNA polymerase II, as well as transcription factors such as estrogen receptor-α (ER). Deregulation of cell cycle and transcriptional control are general features of tumor cells, highlighting the potential for the use of CDK7 inhibitors as novel cancer therapeutics.

Experimental Design—mRNA and protein expression of CDK7 and its essential cofactors cyclin H and MAT1 were evaluated in breast cancer samples to determine if their levels are altered in cancer. Immunohistochemical staining of >900 breast cancers was used to determine the association with clinicopathologic features and patient outcome.

Results—We show that expressions of CDK7, cyclin H, and MAT1 are all closely linked at the mRNA and protein level, and their expression is elevated in breast cancer compared with the normal breast tissue. Intriguingly, CDK7 expression was inversely proportional to tumor grade and size, and outcome analysis showed an association between CAK levels and better outcome. Moreover, CDK7 expression was positively associated with ER expression and in particular with phosphorylation of ER at serine 118, a site important for ER transcriptional activity.

Conclusions—Expressions of components of the CAK complex, CDK7, MAT1, and Cyclin H are elevated in breast cancer and correlate with ER. Like ER, CDK7 expression is inversely proportional to poor prognostic factors and survival.

Introduction

Cyclin-dependent kinases (CDK) control cell proliferation by regulating entry into and passage through the cell cycle (1). The appropriate action of cell-cycle CDKs is ensured by regulation of their activities through the availability of partner cyclins, interaction with CDK inhibitors (CDKi), and through their phosphorylation. Phosphorylation at a key threonine residue in the activation (T) loop facilitates and/or stabilizes the CDK-cyclin complex (2). In metazoans, T-loop phosphorylation is mediated by the CDK-activating kinase (CAK), a trimeric complex consisting of CDK7, Cyclin H, and the accessory protein, MAT1. Importantly, CDK7 is also required for transcription by phosphorylating the C-terminal heptapeptide repeat domain (CTD) of RNA Polymerase II (PolII), a step that is required for gene promoter release and transcription initiation by PolII. Importantly, CDK7 also modulates regulated gene expression by phosphorylating transcription factors, including p53 (3), retinoid receptors (4, 5), androgen receptor (AR; refs. 6, 7), and estrogen receptor (ER; ref. 8). Ligand-dependent phosphorylation of serine 118 (Ser118), important for ERα function and turnover, is mediated by CDK7 (8).

Deregulation of CDK activity by multiple mechanisms, for example, cyclin upregulation and mutation, and silencing or loss of genes encoding CDKis or Rb commonly feature in cancer (9, 10). Hence, the development of inhibitors of cell-cycle CDKs for cancer treatment has received considerable attention, and numerous small-molecule inhibitors have been described (11). Surprisingly, genetic studies have indicated that cell-cycle CDKs, with the exception of CDK1, are not essential for most cell types (12, 13). Nevertheless, following an

initial disappointment with several candidate drugs, newer CDK-selective inhibitors have offered renewed optimism in the utility of these targets. In particular, CDK4/6-selective inhibitors have shown promise against a broad range of cancers, including breast cancer, but can be ineffective, for example, where Rb is absent or inactivated (14, 15). In addition, CDK4/6 inhibitors are efficacious in combination with hormone therapies, for the treatment of ERα-positive advanced breast cancer (16).

Transcription inhibition appears to be important for the antitumor activities of several broad range small molecule inhibitors of CDKs, such as flavopiridol and seliciclib, which inhibit CDK7 and CDK9 (phosphorylation of PolII by CDK9 is needed for transcription elongation), in addition to inhibiting other CDKs. The action of these drugs has been linked to a reduction in PolII phosphorylation and reduced expression of short-lived antiapoptotic proteins, such as Mcl-1 and XIAP, to promote apoptosis (15). The dual role of CDK7 in transcription and the cell cycle means that CDK7 inhibitors potentially provide a potent means of blocking cell-cycle progression, together with the promotion of apoptosis by transcription inhibition in cell lines from a variety of cancer types, including breast, leukemia, neuroblastoma, and lung (17–20). In the latter tumor types, the effects of CDK7 on RUNX1 and MYC expression and function are critical factors in the action of CDK7 inhibition. A further reason for CDK7 as a cancer target is that, although required for early embryonic development, CDK7 was not found to be essential in adult tissues with low proliferative indices (21), indicating that CDK7-selective inhibitors might not show general toxicity in cancer patients.

We have investigated the expression of CDK7 in breast cancer, because this might further support the case for the use of CDK7-selective inhibitors for cancer therapy, particularly in this tumor type. By profiling expression of the components of the CAK complex, CDK7, Cyclin H, and MAT1 in the normal and malignant breast, we demonstrate that their expression is coordinately elevated in breast cancer, especially in ER-positive tumors, compared with normal breast tissue. We also show that CDK7, cyclin H, and MAT1 expression is correlated with ER levels and is related to a good patient prognosis.

Materials and Methods

Breast cancer samples

Tumor and surrounding normal tissue: Tissue samples (snap frozen) were obtained from patients undergoing breast surgery between 2011 and 2013; all patients gave their consent according to the tissue bank protocol (see below). Samples of tumor tissue and surrounding morphologically normal tissue, taken >5 cm from the tumor, were obtained from each patient. All samples were obtained from the Barts Cancer Institute Breast Tissue Bank and were covered by Research Tissue Bank Ethics Approval. RNA was also prepared from tumors from 74 patients who presented with primary, operable breast cancer to the Dundee Cancer Centre between 1997 and 2012 and provided written, informed consent for research use of their tissues; the Tayside Tissue Bank under delegated authority from the Tayside Local Research Ethics Committee approved the use of the clinical material and data. ER immunohistochemical staining and scoring were carried out as described (22). Tissue microarrays (TMA) were prepared from a series of primary operable breast cancer

carcinoma cases from 1986 to 1999 aged 70 years or less at the Nottingham Breast Unit. Patient selection and treatment details have been reported previously (23, 24).

RNA preparation and quantitative RT-PCR

A total of 50 to 100 mg of frozen tissue was homogenized using TissueLyser (Qiagen) with stainless steel ball bearings (5 mm) in 0.7 mL of lysis/binding buffer and the total RNA extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA purity and concentration were measured using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies). cDNA was prepared by reverse transcription of 2.0 μg of total RNA, in a final volume of 20 μL using RevertAid M-MuLV reverse transcriptase (Fermentas) and random hexamer oligonucleotide priming. Quantitative gene expression analysis was carried out using real-time PCR and Taqman gene expression assays for CDK7 (Hs00361486_m1), Cyclin H (Hs00236923_m1), MAT1 (Hs01041574_m1), and GAPDH (Hs99999905_m1; Life Technologies). Gene expression was normalized to GAPDH expression using the $2^{-\Delta\Delta CT}$ method (25).

Gene expression and correlation analysis of microarray data

Expression of CDK7, Cyclin H, and MAT1 was analyzed in normal breast (n = 144) and breast cancer (n = 1,556) samples from the METABRIC dataset using Oncomine (26). For the expression of ER, CDK7, Cyclin H, MAT1, and PGR in the METABRIC dataset of patient samples (n = 1,959), median expression was used as the cutoff in a Cox regression analysis. Kaplan–Meier survival plot, HR with 95% confidence intervals, log-rank P value, and correlation scores (Pearson and Spearman) were calculated and plotted in R using Bioconductor packages.

Immunohistochemistry

Mouse monoclonal antibodies for CDK7 (ab115181; Abcam), cyclin H (ab54903; Abcam), and MAT1 (sc-135981; Santa Cruz Biotechnology) were used for immunohistochemistry (IHC) at a working dilution of 1:100 in Leica antibody diluent. The staining methodology has been described previously (24). Immunohistochemical detection of ER phosphorylated at serine 118 (P-Ser118) was carried out as detailed before (27). Immunostaining was assessed using the H-scoring method, and the X-tile software (28) was used to produce cutoff points for low and high expression levels, as described (24). In brief, this involved random assignment of the patient cohort into two separate training and validation groups ranked by the patient follow-up time. Checking the obtained cutoff points to the validation set tested statistical significance. IHC and scoring for all other proteins have previously been described (29, 30).

Statistical analyses

Statistical analysis of IHC scores for the breast cancer TMAs was performed using SPSS 21 software (SPSS Inc.). The association between CDK7, Cyclin H, and MAT1 and clinicopathologic parameters was determined using the Pearson χ^2 test. Survival curves were estimated by the Kaplan–Meier method with a log-rank test to assess the significance.

Multivariate Cox proportional hazard regression models were used to determine independent prognostic effect of variables.

siRNA transfections

MCF-7 cells were transfected with siRNA using the lipofectamine RNAiMAX reverse transfection protocol (Life Technologies), as described previously (31). siRNAs for CDK7 (s2829, s2830), Cyclin H (s2537 and s2538), MAT1 (s8898 and s8900), and nontargeting (control) siRNA (4390844) were obtained from Life Technologies. Forty-eight hours after transfection, cells were lysed in RIPA buffer. Immunoblotting was carried out using antibodies for CDK7 (ab9516), Cyclin H (ab54903), and ß-actin (ab6276), purchased from Abcam, as described previously (31). Antibodies for MAT1 (sc-13598), TBP (sc-421), and P-Ser118 (sc-12915) were purchased from Santa Cruz Biotechnology and ER (VP-E614) from Vector Laboratories. For performing qRT-PCR, MCF-7 cells were transfected with siRNAs for CDK7, Cyclin H, and MAT1, purchased from Dharmacon. Total RNA was prepared using the RNeasy Kit according to the manufacturer's methods (Qiagen).

Results

CDK7, Cyclin H, and MAT1 are overexpressed in breast cancer

To compare CDK7 expression in normal and malignant breast tissue, we prepared total RNA from 20 breast cancers and matched adjacent normal breast tissue (Fig. 1A–C; Supplementary Fig. S1A–S1C). CDK7 was detectable in all samples at the mRNA level. Interestingly, the majority of tumors were characterized by higher CDK7 expression, compared with the matched adjacent normal tissue. Mean CDK7 expression was 2.2-fold higher in tumors, compared with the adjacent normal tissue ($P = 0.006$). Cyclin H and MAT1 expression was similarly elevated in breast cancer, Cyclin H ($P = 0.0061$) and MAT1 ($P = 0.0057$) levels in tumors being 1.9- and 2.1-fold higher, respectively, than in the normal breast. Epithelial cell adhesion molecule (EpCAM) is an epithelial marker, the expression of which can be elevated in breast cancer (32). EpCAM mRNA levels were not significantly different in our samples (Supplementary Fig. S1D), indicating that the elevated CDK7 expression in this series is unlikely to be due to lower epithelial cellularity of the adjacent normal tissue. IHC of a small series of breast cancer samples showed that nuclear CDK7 immunostaining intensity was consistently higher in tumor cells, compared with CDK7 levels in adjacent normal elements (Supplementary Fig. S1E and S1F). Cyclin H levels were also elevated in tumor cells compared with adjacent normal elements. However, MAT1 levels were not different between normal and cancer cells.

We next analyzed CDK7, Cyclin H, and MAT1 expression in the METABRIC microarray dataset of 1,556 breast cancers and 144 normal breast samples (33). As observed by qRT-PCR in our samples, CDK7 ($P = 1.49 \times 10^{-38}$), Cyclin H ($P = 9.41 \times 10^{-4}$), and MAT1 ($P = 9.06 \times 10^{-10}$) expression was also elevated in breast cancer, compared with expression in normal breast in this data set (Fig. 1D).

Interestingly, these analyses indicated that expression of CDK7, Cyclin H, and MAT1 may be coregulated (for example, see patient samples 1, 2, 10, and 11; Supplementary Fig. S1A–

S1C). Pairwise comparison using Pearson correlation coefficient analysis showed that the expression of CDK7 and Cyclin H is indeed strongly associated in this tumor series (r^2 = 0.861; P < 0.0001), as is the expression of CDK7 and MAT1 (r^2 = 0.879; P < 0.0001) and Cyclin H and MAT1 (r^2 = 0.862; P < 0.0001; Supplementary Fig. S2A–S2C). In agreement with this, Pearson correlation coefficient analysis of the 1,959 samples in the METABRIC cohort showed evidence of a relationship between expression of CDK7 and Cyclin H (r^2 = 0.28), CDK7 and MAT1 (r^2 = 0.25), and an especially strong association between Cyclin H and MAT1 expression (r^2 = 0.69; Supplementary Fig. S2D). The difference in strength of associations in our cohort and METABRIC may reflect, at least in part, differences in proportions of different breast cancer subtypes. Indeed, 63% (47/74) of tumors in our cohort are ER-positive, compared with 77% (1,489/1,928) of the samples in METABRIC. Analysis of The Cancer Genome Atlas and other breast cancer data sets showed that mutations, amplification, and/or deletion of the CDK7, cyclin H, and/or MAT1 genes are uncommon (Supplementary Fig. S2E), so their elevated expression and/or coregulation are unlikely to be the result of gene rearrangement.

To determine if coregulation of the CDK7, Cyclin H, and MAT1 genes can be confirmed experimentally in breast cancer cells, we performed siRNA for CDK7 in MCF-7 cells. Efficient CDK7 knockdown was achieved at both the mRNA and protein levels (Fig. 1E and H). In addition, siCDK7 transfection also resulted in Cyclin H and MAT1 downregulation at the mRNA and protein levels (Fig. 1F–H). Similarly, transfection of MCF-7 cells with Cyclin H siRNA led to reductions in Cyclin H, but also reductions in the levels of CDK7 and MAT1 mRNA and protein. Finally, MAT1 siRNA reduced not only MAT1, but also CDK7 and Cyclin H expression. By contrast, expression of the TFIIH p62 subunit was unaffected, as were TBP and ER levels, suggesting that the siRNA-mediated reduction in CAK expression is specific. In agreement with our findings, reduction in protein levels of all three CAK subunits has been reported for CDK7 and MAT1 knockout mice (21, 34). What is striking from our results is that knockdown of one CAK subunit not only results in reduction in protein levels of the other subunits, which might be attributable to disruption of the CAK complex, rather mRNA levels of the other subunits are reduced, implicating transcriptional or posttranscriptional mechanisms in the co-ordinate regulation of CAK subunit mRNA levels. Finally, immunoblotting of MCF7 cells sorted by flow cytometry showed similar expression patterns for CDK7, cyclin H, and MAT1 through the cell cycle, with highest levels of each subunit in G_1 and G_2–M (Supplementary Fig. S3A and S3B). siRNA-mediated knockdown of CDK7 was not associated with cell-cycle arrest, but resulted in apoptosis (Supplementary Fig. S3C), as has been described for CDK7 inhibitors BS-181 and THZ1 (17, 18).

CDK7, Cyclin H, and MAT1 expression is associated with better patient outcome in breast cancer

In order to determine expression of the CAK complex proteins in breast cancer and to analyze associations with clinical features, we carried out IHC of breast cancer TMAs for CDK7 (n = 945), Cyclin H (n = 1,218), and MAT1 (n = 910; Fig. 2A). Differential staining was evident for different samples, so the H-scoring method and cutoff H-scores were used to segregate tumors into high and low expression groups. Spearman Rank correlation of protein

levels for the CAK subunits (H-scores) showed that expression of CDK7, Cyclin H, and MAT1 is strongly associated ($P < 0.001$; Fig. 2B), as was observed for mRNA levels, further evidence for an important relationship between expression of the three CAK subunits in breast cancer.

There was a suggestion of an association of CDK7 expression with age in patients ages between 51 and 60 ($P = 0.042$), but not with menopausal status ($P = 0.39$; Table 1). High MAT1 levels were also weakly associated with age ($P = 0.044$), but not with menopausal status ($P = 0.22$). Importantly, elevated expression of CDK7, Cyclin H, and MAT1 was associated with markers of better prognosis. Hence, patients with high grade and larger tumors, or those who developed recurrent disease, featured low CDK7, Cyclin H, and/or MAT1 expression. As expected from the association of high levels of the CAK components with low tumor grade and reduced recurrence, high CDK7 expression was associated with longer breast cancer–specific survival (BCSS; log-rank = 4.11, $P = 0.04$; Fig. 2C). Multivariate Cox hazard analysis, including tumor stage, size, grade, and lymph node (LN) status, showed an increased significance of CDK7 with longer BCSS [HR, 0.65 (0.41–0.84), $P = 0.001$; Table 2]. Multivariate analysis also showed a benefit for high CDK7 expression for time to distant metastasis (TTDM; Supplementary Table S1). High Cyclin H (log-rank = 7.32; $P = 0.007$) and MAT1 (log-rank = 8.43; $P = 0.004$) were also associated with longer BCSS. Univariate analysis for low or high expression of all three CAK subunits maintained the survival benefit (log-rank = 6.09; $P = 0.014$). In the multivariate analysis model, tumor size, LN status, and HER2 status, along with BCSS, were significantly associated with high Cyclin H ($P = 0.003$) and MAT1 expression ($P = 0.016$), as was high expression of all three proteins (CAK; $P = 0.001$; Table 2; Supplementary Table S1).

Expression levels of the CAK subunits are associated with ER expression in breast cancer

Interestingly, CDK7 ($P = 0.001$), Cyclin H ($P < 0.001$), and MAT1 ($P < 0.001$) levels were higher in ER-positive than in ER-negative breast cancer (Table 3), and there was a positive association of CDK7 ($P = 0.002$), Cyclin H ($P < 0.001$), and MAT1 ($P < 0.001$) levels with PGR positivity, together indicative of higher CAK levels in luminal breast cancer. This was further confirmed by the fact that CDK7, cyclin H, and MAT1 levels were associated with AR positivity, as AR expression is strongly associated with ER (35, 36). There was also a significant association with the luminal A marker, GATA3 (37), although there was an inverse relationship with FOXA1, another important marker of luminal A breast cancer. Indeed, CDK7, Cyclin H, and MAT1 levels were lower in HER2-positive than in HER2-negative breast cancer and in triple-negative (TN) breast cancer, compared with non-TN breast cancer.

Real-time RT-PCR analysis of RNAs prepared from 74 independent breast cancers showed that CDK7 mRNA levels are also positively associated with ER mRNA levels ($r^2 = 0.56$; $P < 0.0001$; Supplementary Fig. S4A), as are Cyclin H ($r^2 = 0.46$; $P < 0.0001$) and MAT1 ($r^2 = 0.44$; $P < 0.0001$) mRNA levels. As expected, a relationship between ER and PGR mRNA levels ($r^2 = 0.63$; $P < 0.0001$) was also observed in this patient series. The association was also evident when comparing CDK7, Cyclin H, and MAT1 mRNA levels with immunohistochemically defined ER status in this sample set (Supplementary Fig. S4B and

S4C). Further confirming this association, CDK7, Cyclin H, and MAT1 mRNA expression was also positively associated with ER mRNA levels in the METABRIC dataset (Supplementary Fig. S4D). Analysis of the CAK expression in the PAM50 breast cancer subtypes showed slightly higher expression in luminal B than in luminal A breast cancer; P = 0.03, 0.02, and <2.2e–16 for CDK7, cyclin H, and MAT1, respectively (Supplementary Fig. S4E–S4G). Moreover, CAK expression was significantly higher in luminal A/B than in $HER2^+$ or in basal breast cancer. Interestingly, expression of each of the CAK subunits was also higher in HER2-positive than in basal breast cancer.

Taken together, the simplest explanation for the high expression of CAK in ER-positive breast cancer is that ER regulates their expression. However, treatment of MCF-7 cells with estrogen did not affect expression of any of the subunits (Supplementary Fig. S5A). Furthermore, ER knockdown did not affect CAK levels (Supplementary Fig. S5B). Examination of ER ChIP-seq for MCF7 cells (38), did not identify ER binding regions within the genes, nor within 50 kb 5′ or 3′ to the CDK7, Cyclin H or MAT1 genes. Together, these results indicate that the association between expression of the CAK genes and ER expression is not due to direct regulation by ER. Interestingly, high expression of the CAK complex in our TMA series was associated with longer TTDM (log-rank = 6.68; P = 0.01) and BCSS in ER-positive breast cancer (log-rank = 5.61; P = 0.018; Fig. 2D and E).

Evidence for a role of CDK7 in phosphorylation of ER at serine 118 in breast cancer

Phosphorylation of ER at serine 118 (Ser118) promotes ER activity, CDK7 has been shown to mediate ligand-dependent phosphorylation of Ser118 (8, 39), and CDK7 knock-down resulted in reduction in Ser118 phosphorylation (Fig. 1H). To determine if Ser118 phosphorylation is related to CDK7 expression in breast cancer, we performed IHC for ER phosphorylated at Ser118 (P-Ser118). In agreement with previous findings linking Ser118 phosphorylation with better prognosis (40), Ser118 phosphorylation was associated with better TTDM and BCSS, high P-Ser118 levels being correlated with better survival. Patients with intermediate P-Ser118 had worse prognosis, and patients with very low/absent P-Ser118 had the poorest survival (TTDM log-rank = 19.9, P < 0.001; BCSS log-rank = 13.0, P = 0.005; Supplementary Fig. S3).

More than half (54.1%) of the CDK7-low breast cancers were negative or were weakly positive for P-Ser118, compared with just 26.2% of the CDK7-high tumors (P < 0.001; χ^2 = 56.3; Table 4). As expected, similarly strong associations were obtained for P-Ser118 and Cyclin H (P < 0.001; χ^2 = 43.8), and P-Ser118 and MAT1 (P < 0.001; χ^2 = 66.5).

Discussion

The importance of CDK7 in cell-cycle regulation and transcription has highlighted this kinase as a potential therapeutic target for cancer treatment. In line with this, recently described CDK7-selective inhibitors show antitumor activity in several cancer models (17–20). Importantly, these studies show that transcriptional drivers that are especially important in specific cancer types, for example, RUNX1 in leukemia, are particularly sensitive to CDK7 inhibition (18). Similarly, particular sensitivity of the MYCN (neuroblastoma; ref. 20) and MYC (lung cancer; ref. 19) genes to CDK7 inhibition has been described and

appears to be due to hypersensitivity of super-enhancers that drive expression of these factors. Given this diverse range of tumor types that potentially respond, we wanted to determine if CDK7 expression is altered in cancer, as expression and activity may be important factors in the utility of CDK7 inhibitors in the clinic. We chose to investigate CDK7 expression in breast cancer, since we have previously shown that a selective CDK7 inhibitor, BS-181, inhibits breast cancer cell growth *in vitro* and *in vivo* (17). Moreover, CDK7 directly regulates the transcriptional activity of ER by phosphorylating Ser118 (8, 39); thus, CDK7 inhibitors might be especially effective in ER-positive breast cancer featuring elevated Ser118 phosphorylation.

Comparison of breast cancers with matched adjacent normal tissue showed that CDK7 mRNA levels are elevated in this tumor type. This was confirmed by analysis of microarray datasets and is in agreement with previous reports which suggested that CDK7 protein levels are higher in cancer compared with the normal breast (41, 42). It is possible that these observations reflect differences in epithelial cell content. However, real-time RT-PCR for EpCAM, as well as comparison of CDK7 IHC for normal breast with CDK7 levels in tumor samples, indicates that CDK7 levels are indeed elevated in breast cancer. Mutations and gene rearrangements at the CAK gene loci are uncommon, so this is unlikely to represent a major mechanism for high expression in breast cancer. Interestingly, the CDK7 and Cyclin H genes are located 18 Mb apart on human chromosome 5 and are also linked in the genomes of several other vertebrates, including Zebrafish, chicken, rodents, and man, making coregulation through common gene regulatory elements possible.

Remarkably, we observed that mRNA and protein levels of Cyclin H and MAT1, both of which are required for CDK7 activity, are also increased in breast cancer, indicative of upregulation of CAK activity in breast cancer. siRNA experiments also showed that knockdown of any one CAK subunit resulted in reduced expression of the other subunits, implying coregulation of the expression of the CAK complex at the transcriptional level. Although the exact mechanisms underlying this coregulation remain unclear, treatment with the CDK7 inhibitor BS-181 resulted in reduced CDK7 protein levels (17) and THZ1, a covalent CDK7 inhibitor, inhibits PolII recruitment to gene promoters (18), indicating that expression of the CAK complex is strongly linked to CDK7 activity. It is possible that this coregulation is due to loss of CAK subunits in apoptotic cells. Notwithstanding, IHC staining of >900 breast cancers also demonstrated a significant association between levels of CDK7, Cyclin H, and MAT1, which, together with the association for mRNA expression of the CAK subunits, provides strong evidence for coregulation of CAK subunit expression in breast cancer.

Interestingly, high-level expression of each of the CAK subunits was associated with longer survival in univariate and multivariate analyses. The relationship between CDK7 and prognosis in breast cancer seems analogous to the relationship between ER and prognosis, in that ER confers a good prognosis, but is at the same time a suitable target for therapy. Moreover, the majority of tumors with high CDK7 levels were ER-positive, as were tumors with high levels of Cyclin H and MAT1. In agreement with this, real-time RT-PCR and analysis of the METABRIC microarray datasets showed positive associations between mRNA levels of each CAK subunit with ER mRNA levels, as well as ER status. In ER-

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positive breast cancer, CAK expression was also associated with better prognosis. This does not appear to be due to higher CAK expression in luminal A compared with luminal B breast cancer. Indeed, in METAB-RIC, CAK transcript levels are similar to, or higher in, luminal B than in luminal A breast cancer. The mechanisms underlying the association between CDK7, Cyclin H, and MAT1 expression and ER are unclear, but it is interesting to note that CAK levels were strongly associated with ER phosphorylation at Ser118, which provides *in vivo* evidence for the previously described role of CDK7 in phosphorylating this residue (8). Ser118 phosphorylation is important not only for stimulating ER activity, but also regulates ER degradation and consequently ER levels (43, 44). This might afford a potential explanation for the relationship between levels of CAK and ER protein in breast cancer. Moreover, ER positively regulates its own gene expression, at least in part through a positive cross-regulatory loop with GATA-3 mRNA levels (45). CDK7 may thus promote ER gene expression by stimulating its activity through phosphorylation of Ser118. Alternatively, transcription of the ER gene may be particularly sensitive to CDK7 activity, as demonstrated by the sensitivity of the RUNX1 and MYC genes in other cancers (18).

We previously reported the first specific, small-molecule CDK7 inhibitor, BS-181 (17). We showed that BS181 promotes p53-dependent and independent apoptosis, at least in part by inhibiting the expression of short-lived transcripts for genes encoding inhibitors of apoptosis. The additional work presented here shows that CAK siRNA reduces ER phosphorylation, in line with its known action on ER phosphorylation at Ser118 (8). Our findings, therefore, offer some explanation as to why CDK7 expression carries a good prognosis in patients with ER-positive breast cancer, in that in our sets, these patients have been treated with adjuvant endocrine therapy, where improved survival is dependent on a functioning ER, for which Ser118 phosphorylation is critical.

In summary, CDK7, Cyclin H, and MAT1 mRNA and protein levels are elevated in breast cancer, particularly in ER-positive breast cancer. Given the importance of CDK7 in regulation of transcription, as well as its role in the direct regulation of ER activity through phosphorylation of Ser118, our findings support the potential use of CDK7 inhibitors in the treatment of ER-positive breast cancer, either as a single agent or in combination with hormonal therapy, with perhaps the most suitable group for treatment being ER-positive breast cancer patients with high CDK7 and P-Ser118 levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Cyclin-dependent kinase 7 (CDK7) is a critical regulator of cell-cycle progression and gene expression, processes that are frequently deregulated in cancer. As such, inhibition of CDK7 activity has been proposed as a therapeutic strategy for the treatment of cancer, an aim that is supported by the recent development of selective CDK7 inhibitors with potent anti-cancer activities. Since CDK7 is centrally involved in key cellular processes in all cells, the use of CDK7 inhibitors could be limited by a toxicity associated with its function in normal tissues. Using mRNA expression profiling and immunohistochemistry, we find that expression of CDK7, as well as the associated cofactors Cyclin H and MAT1, are all elevated in breast cancer, suggesting that this tumor type may be especially sensitive to CDK7 inhibition and that the CDK7 overexpression may allow mitigation of toxicity seen in normal tissues.

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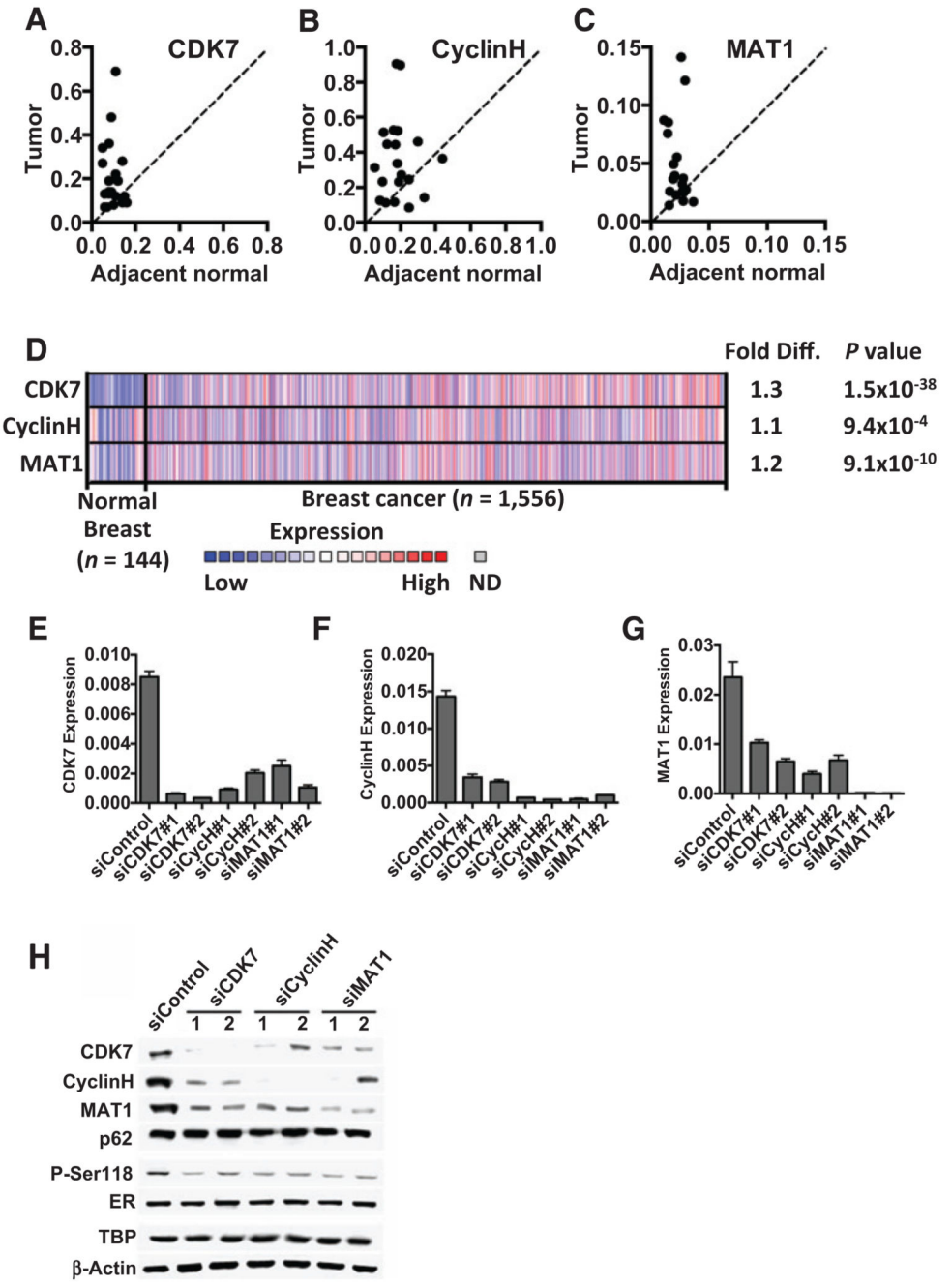

Figure 1.

CDK7, Cyclin H, and MAT1 mRNA levels are elevated in breast cancer and show evidence of coordinate regulation. **A–C**, CDK7, Cyclin H, and MAT1 mRNA levels, determined by real-time RT-PCR analysis, were normalized to the expression of GAPDH for RNA prepared from 20 paired tumor and adjacent normal tissues. **D**, Analysis of microarray data from the METABRIC samples for expression of CDK7, Cyclin H, and MAT1 in normal breast and breast cancer samples. **E–G**, MCF-7 cells were transfected with two independent siRNAs for CDK7, Cyclin H, and MAT1. Real-time RT-PCR was performed using RNA prepared 48

hours after transfection. CDK7, Cyclin H, and MAT1 expression is shown relative to expression of GAPDH for three independent samples. Expression of all three genes was significantly reduced ($P < 0.001$) for each siRNA when compared with the control siRNA. **H**, Immunoblotting was performed using protein lysates prepared following siRNA transfection as above.

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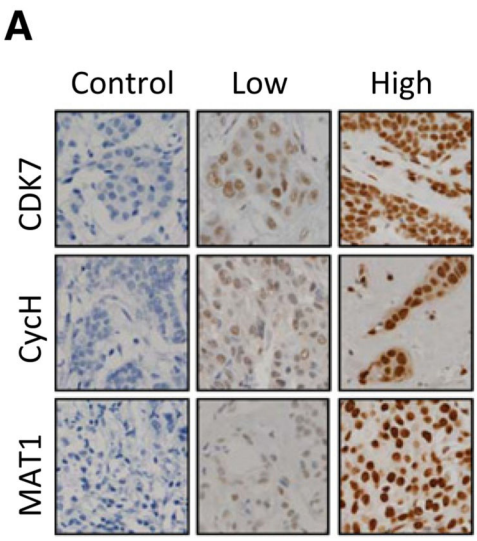

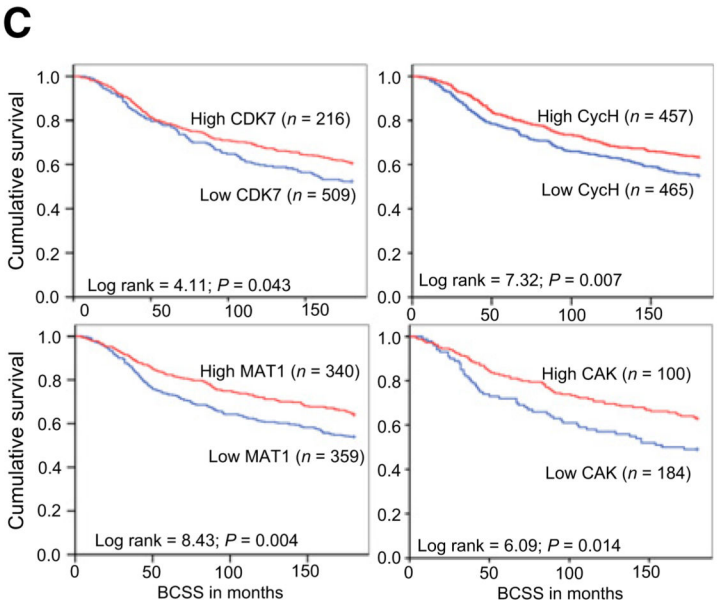

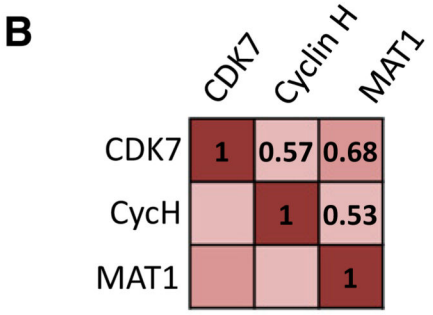

D

Variable	TTDM		
	P	HR	95% CI
Tumor size	0.007	1.81	1.18–2.79
Stage	0.021	1.43	1.06–1.93
Grade	0.157	1.25	0.92–1.71
HER2	0.002	2.63	1.44–4.80
CDK7	0.047	0.65	0.43–0.99

Variable	BCSS		
	P	HR	95% CI
Tumor size	0.002	2.14	1.31–3.49
Stage	<0.001	1.88	1.35–2.61
Grade	0.03	1.50	1.03–2.18
HER2	0.025	2.11	1.10–4.05
CAK	0.016	0.55	0.33–0.89

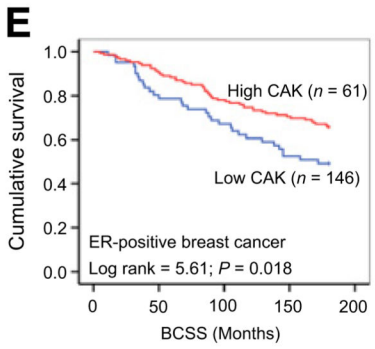

Figure 2.

Immunohistochemical analysis of CDK7, Cyclin H, and MAT1 expression in breast cancer. CDK7, Cyclin H, and MAT1 antibodies were used to immunostain breast cancer TMAs. The sections were scored as CDK7 low (H-score: 0–160), CDK7 high (H-score: 161–300), Cyclin H low (H-score: 0–194), Cyclin H high (H-score: 195–300), MAT1 low (H-score: 0–179), or MAT1 high (H-score: 180–300). Negative controls were performed by omitting the primary antibody. **A**, Staining representative of low and high H-scores is shown. **B**, Pearson's correlation analysis is shown, together with r^2 values for each pair-wise

comparison. **C**, Kaplan–Meier plots showing BCSS for CDK7, Cyclin H, and MAT1 expression in breast cancer. **D**, COX regression analysis for TTDM and BCSS for ER-positive breast cancer samples only. **E**, Kaplan-Meier plot of high versus low CAK levels in ER-positive breast cancer.

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Table 1

CDK7, CycH, and MAT1 expression and clinicopathologic associations

	CDK7 expression (%)			CycH expression (%)			MAT1 expression (%)		
Variable	**Low (0 –160)**	**High (161–300)**	***P***	**Low (0–194)**	**High (195–300)**	***P***	**Low (0 –179)**	**High (180 –300)**	***P***
Age									
<40	16 (5.9)	65 (9.7)	0.042	59 (9.7)	46 (7.6)	0.34	38 (8.6)	40 (8.7)	0.044
41–50	72 (26.8)	172 (25.6)		159 (26.2)	167 (27.6)		111 (25.2)	130 (28.1)	
51–60	*100 (37.2)*	*198 (29.5)*		198 (32.6)	182 (30)		*158 (35.8)*	*126 (27.3)*	
>60	81 (30.1)	236 (30.1)		192 (31.6)	211 (34.8)		*134 (30.4)*	*166 (35.9)*	
Menopausal status									
Pre	100 (37.6)	259 (38.9)	0.39	237 (39.4)	238 (39.5)	0.97	162 (37.1)	189 (41.1)	0.22
Post	166 (62.4)	407 (61.1)		365 (60.6)	365 (60.5)		275 (62.9)	271 (58.9)	
Tumor size (cm)									
<2.0	121 (44.8)	326 (48.9)	0.28	273 (45)	307 (50.6)	0.051	*185 (42)*	*246 (53.7)*	<0.001
≥2.0	149 (55.2)	341 (51.1)		334 (55)	300 (49.4)		*255 (58)*	*212 (46.3)*	
Grade									
1	36 (13.4)	98 (14.7)	0.16	*84 (13.9)*	*109 (18)*	<0.001	*53 (12.1)*	*85 (18.6)*	<0.001
2	82 (30.5)	239 (35.9)		*174 (28.7)*	*229 (37.9)*		*118 (26.9)*	*176 (38.4)*	
3	151 (56.1)	328 (49.3)		*348 (57.4)*	*266 (44)*		*268 (61)*	*197 (43)*	
LN									
Negative	*186 (68.9)*	*382 (57.2)*	0.001	382 (62.9)	363 (59.8)	0.26	*286 (65)*	*264 (57.5)*	0.021
Positive	*84 (31.1)*	*286 (42.8)*		225 (37.1)	244 (40.2)		*154 (35)*	*195 (42.5)*	
Local recurrence									
No	210 (79.2)	555 (84.3)	0.07	501 (84.1)	486 (82.5)	0.48	355 (81.8)	376 (83.2)	0.59
Yes	55 (20.8)	103 (15.7)		95 (15.9)	103 (17.5)		79 (18.2)	76 (16.8)	
Regional recurrence									
No	*212 (80)*	*576 (77.5)*	0.003	510 (85.6)	516 (87.6)	0.3	*351 (80.9)*	*399 (88.3)*	0.002
Yes	*53 (20)*	*82 (22.5)*		86 (14.4)	73 (12.4)		*83 (19.1)*	*53 (11.7)*	
Distant metastasis									
No	*149 (55.2)*	*417 (62.4)*	0.04	373 (61.6)	388 (64.1)	0.35	*251 (56.9)*	*304 (65.9)*	0.005
Yes	*121 (44.8)*	*251 (37.6)*		233 (38.4)	217 (35.9)		*190 (43.1)*	*157 (34.1)*	

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Table 2

Multivariate Cox regression analysis for BCSS

Variable	*P*	HR (95% CI)
Tumor size	0.002	1.48 (1.15–1.90)
Stage	<0.001	1.97 (1.65–2.35)
Grade	<0.001	1.78 (1.45–2.19)
HER2	<0.001	1.69 (1.26–2.26)
CDK7	0.001	0.65 (0.51–0.84)
Tumor size	0.001	1.49 (1.18–1.88)
Stage	<0.001	1.91 (1.65–2.23)
Grade	<0.001	1.85 (1.54–2.23)
HER2	<0.001	1.67 (1.28–2.20)
CycH	0.003	0.72 (0.58–0.90)
Tumor size	0.049	1.28 (1.01–1.63)
Stage	<0.001	1.82 (1.53–2.16)
Grade	0.001	1.38 (1.15–1.66)
HER2	0.001	1.66 (1.24–2.23)
MAT1	0.016	0.75 (0.59–095)
Tumor size	0.002	1.82 (1.25–2.65)
Stage	<0.001	1.95 (1.50–2.53)
Grade	0.10	1.29 (0.95–1.76)
HER2	0.002	2.09 (1.32–3.32)
CAK	0.001	0.53 (0.37–0.78)

Table 3

Relationship between CDK7, Cyclin H, and MAT1 expression and breast cancer subtypes

	CDK7			Cyclin H			MAT1		
Variable	**Low (0–160)**	**High (161–300)**	***P*** **(χ^2)**	**Low (0–194)**	**High (195–300)**	***P*** **(χ^2)**	**Low (0–179)**	**High (180–300)**	***P*** **(χ^2)**
ER									
Negative	87 (32.2)	148 (22.2)	0.001	194 (32.1)	117 (19.4)	<0.001	151 (34.2)	93 (20.3)	<0.001
Positive	183 (67.8)	520 (77.8)	(10.4)	410 (67.9)	486 (80.6)	(25.5)	291 (65.8)	365 (79.7)	(21.9)
PGR									
Negative	128 (49.2)	248 (38.3)	0.002	292 (49.6)	191 (33)	<0.001	214 (49.7)	157 (35.7)	<0.001
Positive	132 (50.8)	400 (61.7)	(9.2)	297 (50.4)	388 (67)	(33.1)	217 (50.3)	283 (64.3)	(17.4)
ER/PGR status									
ER^+/PGR^+	132 (50.8)	400 (61.7)	0.003	295 (50.3)	387 (67.1)	<0.001	217 (50.3)	282 (64.4)	<0.001
ER^+/PGR^-	46 (17.7)	108 (16.7)	(11.4)	104 (17.7)	80 (13.9)	(35.4)	68 (15.8)	71 (16.2)	(25.6)
ER^-/PGR^-	82 (31.5)	140 (21.6)		187 (31.9)	110 (19.1)		146 (33.9)	85 (19.4)	
AR									
Negative	125 (52.7)	199 (33.2)	<0.001	249 (46.5)	155 (28.9)	<0.001	179 (46.9)	120 (29.3)	<0.001
Positive	112 (47.3)	400 (66.8)	(27.3)	287 (53.5)	381 (71.1)	(35.1)	203 (53.1)	290 (70.7)	(26.0)
FOXA1									
Negative	67 (34.7)	209 (48.5)	0.001	154 (37.6)	199 (52.5)	<0.001	119 (36.1)	150 (52.8)	<0.001
Positive (≥10)	126 (65.3)	222 (51.5)	(10.3)	256 (62.4)	180 (47.5)	(17.8)	211 (63.9)	134 (47.2)	(17.4)
GATA3									
Negative/low (<60)	154 (87.5)	277 (71.9)	<0.001	310 (83.8)	229 (70.2)	<0.001	245 (86)	177 (67.3)	<0.001
Positive (≥60)	22 (12.5)	108 (28.1)	(16.4)	60 (16.2)	97 (29.8)	(18.2)	40 (14)	86 (32.7)	(26.9)
HER2									
Negative	212 (81.2)	566 (87.5)	0.015	506 (86.3)	500 (86.8)	NS	350 (81.4)	394 (90)	<0.001
Positive	49 (18.8)	81 (12.5)	(5.9)	80 (13.7)	76 (13.2)		80 (18.6)	44 (10)	(13.0)
TN									
Non-TN	207 (87.7)	554 (84.6)	0.033	453 (76.3)	523 (88.5)	<0.001	329 (76.3)	390 (87.1)	<0.001
TN	56 (21.3)	101 (15.4)	(4.6)	141 (23.7)	68 (11.5)	(30.5)	102 (23.7)	58 (12.9)	(16.9)

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Table 4

Phosphorylation levels of ER Serine 118 are associated with CDK7, CycH, and MAT1 levels

Variable	P-Ser118-ER				
	Negative (0–50)	Low (51–100)	Moderate (101–200)	High (201–300)	*P* (χ^2)
CDK7					
Low (0–160)	64 (43.8)	15 (10.3)	51 (34.9)	16 (11.0)	<0.001
High (161–300)	63 (15.3)	45 (10.9)	182 (44.1)	123 (29.8)	(56.3)
CycH					
Low (0–194)	81 (36.0)	31 (13.8)	74 (32.9)	39 (17.3)	<0.001
High (195–300)	68 (15.3)	47 (10.6)	197 (44.4)	132 (29.7)	(43.8)
MAT1					
Low (0–179)	84 (35.7)	30 (12.8)	97 (14.3)	24 (10.2)	<0.001
High (180–300)	38 (13.6)	27 (9.6)	109 (38.6)	106 (37.9)	(66.5)